

**Peptide Expression and Purification to Elucidate the Mechanism of
Vascular α_2C -Adrenoceptor Translocation**

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By

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Published Abstract

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Abstract

In response to physiological cold temperatures, cutaneous blood vessels constrict to reduce heat loss. This constriction is mediated by smooth muscle G protein-coupled α_{2C} -adrenoceptors (α_{2C} -ARs), which translocate from the trans-Golgi to the plasma membrane upon cooling. The cellular mechanisms involved in receptor translocation are not fully understood; however, this translocation is hypothesized to be dependent upon a protein-protein interaction between the α_{2C} -AR C-terminus and the protein filamin-2. This portion of the receptor translocation mechanism can be tested experimentally by decoy peptide interference.

The decoy peptide contains a TAT domain and an identical C-terminus to the α_{2C} -AR, allowing the peptide to penetrate the cell membrane and mimic the α_{2C} -AR, respectively. We predict that interfering with this interaction will inhibit translocation of α_{2C} -ARs and subsequent receptor function. Therefore, the goal of this study is to generate and purify a decoy peptide, confirm delivery to human VSM cells, and assess cellular activity for endogenous α_{2C} -ARs.

The DNA sequence encoding 11 amino acids of the cell-permeable HIV transactivator of transduction (TAT) domain was fused in frame with the α_{2C} -AR C-terminus to generate the p-TAT- α_{2C} -AR-C-terminus bacterial expression vector. BL21-CodonPlus competent cells were used to optimize peptide expression at 30°C. The

parameters tested during bacterial growth included the presence of antibiotics, induction time, and growth temperature. Additionally, cell lysis, sonication and salt precipitation conditions were optimized. Peptide expression was confirmed by Western blot and Coomassie techniques. The peptide was purified by ammonium sulfate, ion-exchange chromatography and nickel column techniques. The peptide was successfully delivered to human VSM and HEK-293 cells, confirmed by microscopic visualization and western blot techniques, which will be utilized to assess activity of endogenous α_2C -ARs. Additionally, these studies have broader implications for understanding the role of actin in protein translocation and cell surface delivery.

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Chapter 1: Introduction

1.1 α_{2C} -Adrenoceptors (α_{2C} -ARs)

Vascular smooth muscle cells, such as those found in arteries, arterioles and veins, are constriction regulated by a family of G protein coupled receptors known as α_2 -adrenoceptors (α_2 -ARs). These receptors are separated into three classifications based on their structure and physiological function, denoted α_{2A} , α_{2B} , and α_{2C} . Previous studies show that the majority of vascular constriction is regulated by α_{2A} and α_{2B} activity (MacDonald, Kobilka and Scheinin). Recent studies indicate that α_{2C} -ARs are responsible for constriction induced specifically by physiologically cold temperatures (Chotani, Mitra and Su). Collectively, experiments correlate high levels of vascular constriction with increased levels of α_2 -AR activity (Chotani, Flavahan and Mitra).

Blood vessels at the periphery of the body conserve heat by constricting upon exposure to cold temperatures, which reduces blood flow to the extremities and therefore an organism's heat loss. During physiologically warm temperature (37°C experimentally), α_{2C} -AR activity is silenced because of its intracellular location in the Golgi apparatus (Jeyaraj, Chotani and Mitra). Upon cold exposure, α_{2C} -ARs trans-locate to the plasma membrane, where they engage with the agonist and are activated. When cold stimulus is removed, the α_{2C} -ARs are no longer functional, and the blood vessels dilate to their relaxed state (Chotani, Flavahan and Mitra).

Certain individuals are prone to vessel dysfunction, causing the vessels to remain constricted. A result of this cellular dysfunction is Raynaud's phenomenon, a disease in which blood flow is reduced to extremities following cold exposure, leading to discoloration, atrophy of the skin, and potentially more severe consequences (Flavahan, Flavahan and Mitra). This vessel dysfunction is likely related to increased α_{2C} -AR activity; therefore, understanding the cellular

mechanism of cold induced blood vessel constriction may lead to a pharmaceutical treatment of this disease.

Sub-cellular protein retention is due to the amino acid motif “RXR”, R being arginine and X being any amino acid. The α_{2C} -AR fits the proposed mechanism with a stretch of amino acids “RRRRR” (Eid, Molecular Regulation of Vascular α_{2C} - Adrenoceptors). However, the α_{2C} -AR is of particular interest because it re-locates to the membrane upon cooling. The mechanism of trans-location is yet to be determined and is the focus of our research.

The α_2 -AR family of proteins contains a trans-membrane spanning domain along with several other conserved amino acid sequences. However, the α_{2C} -AR most significantly differs from the α_2 -AR family of proteins in the C-terminus. Therefore, the α_{2C} -AR C-terminus was utilized in a yeast two-hybrid approach, effectively identifying a novel protein-protein interaction with the actin binding protein, filamin-2 (MA Chotani, unpublished results). The strong affinity of α_{2C} -AR C-terminus to filamin-2 indicates that trans-location may depend upon their interaction, which leads to the utilization of a decoy peptide.

1.2 Decoy Peptide

A decoy peptide serves to mimic the α_{2C} -AR, specifically the C-terminal sequence hypothesized to be responsible for receptor trafficking via binding to filamin-2. To utilize this technique, the cell is loaded with an excess of the decoy peptide, effectively saturating the binding sites to filamin-2 and limiting α_{2C} -AR binding capabilities. The general procedure for obtaining this peptide in an experimentally useful form is outlined by the following steps: DNA cloning, bacterial transformation, bacteria growth, transcription induction, cell lysis, and peptide purification.

During expression of heterologous peptides in *E. Coli*, cells frequently interpret peptides as potentially harmful. As a result, the bacteria form inclusion bodies around the peptide, which are insoluble, micelle-like structures. However, for a successful purification, it is important to express the peptide in a soluble form. The formation of inclusion bodies is a major obstacle because it prohibits soluble expression and purification.

A critical component of the decoy peptide is the TAT domain, which allows the successful transduction of full length proteins into primary and transformed cells. The TAT amino acid sequence is derived from the cell-permeable HIV transactivator of transduction, which is a DNA sequence encoding 11 amino acids (Becker-Hapak, McAllister and Dowdy). The proposed mechanism of cellular entry via the TAT amino acid sequence is macropinocytosis, in which a sequence of highly charged arginine residues binds to heparin sulfate on the cell membrane of an actin protrusion. The process of peptide entry is non-passive, indicating that molecules stimulate their own endocytosis. Not much is known about the degradation of large endosomes inside the cell, however it is likely due to chemical, potential, or pH gradients along the endosome path to the Golgi (Gump and Dowdy).

1.3 Hypotheses

Our hypothesis is that the interaction of the α_{2C} -AR C-terminus with filamin-2 is necessary for its translocation from the Golgi to the cell surface. Interference with α_{2C} -AR binding to filamin-2 will inhibit the localization of α_{2C} -AR to the cell surface and subsequent biological activity.

At warm temperatures (37°C), we expect to see no changes in the activity of α_{2C} -AR. Upon cooling, interference with filamin-2 will reduce the re-localization of α_{2C} -AR to the cell surface compared to control cells without the decoy peptide.

Chapter 2: Experimental Methods

2.1 DNA Cloning

The α_{2C} -AR C-terminus was amplified via polymerase chain reaction (PCR) using primers with restriction enzyme insertion sites at XhoI and EcoRI. The decoy peptide was expressed using a pTAT-HA bacterial expression vector, which contains the lac promoter, a poly-histidine sequence for purification, the TAT domain for cellular transduction, and an HA tag for labeling. A visual representation of the plasmid DNA encoding the decoy peptide of interest can be seen in Figure 1, and the amino acid sequence of the decoy peptide's C terminus can be seen in Figure 2.

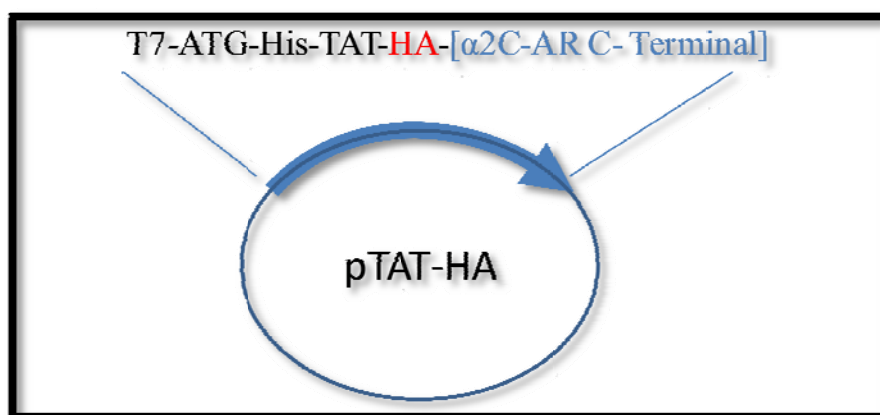


Figure 1: pTAT-HA- α_{2C} -AR Plasmid Vector

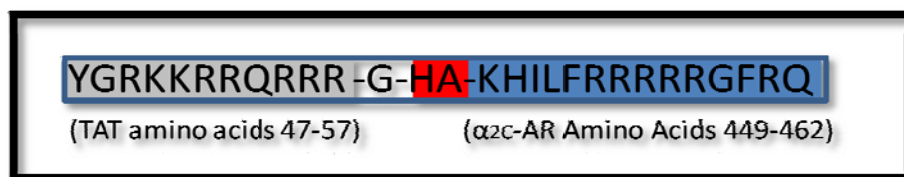


Figure 2: Decoy Peptide C Terminus

2.2 Bacterial Transformation

In the following transformation protocol, 25 nano-grams (ng) plasmid DNA was added to the competent cell solution. This parameter can be adjusted depending on the desired yield and

efficiency. Maximum transformation efficiency is observed using 0.1 ng DNA, and maximum yield is observed when using 50 ng DNA.

BL-21 CodonPlus (DE3)-RIPL competent cells were used for transformation because they contain extra copies of tRNAs that enhance expression of heterologous proteins. The transformations were performed according to manufacturer recommended protocol. The entirety of the transformation protocol can be seen in the instruction manual for BL21 Competent Cells (Stratagene, Cat # 200131 Rev #066004e).

The plasmid DNA and competent cell solution set on ice for 30 minutes, which allowed the diffusion of DNA into the permeated membrane. To close the cell membrane, the cells were heat pulsed at 42°C for 20 seconds and placed on ice for 2 minutes. The cells were grown in pre heated SOC medium (42°C), and finally the cells were spread onto LB agar plates with the antibiotics ampicillin and chloramphenicol. The pTAT-HA expression vector and the competent cell host strain contained genes encoding ampicillin and chloramphenicol resistance, respectively. The dual antibiotic treatment allowed selective growth of bacteria that contained genes encoding the decoy peptide and transcription factors. The successfully transformed bacteria colonies were made into glycerol stocks for long term storage. To create glycerol stocks, equal volumes of the bacteria solution and 50% sterile glycerol were mixed, vortexed, and stored at -80 °C.

2.3 Bacteria Growth

Bacteria growth was performed using freshly transformed bacteria colonies or glycerol stocks once their peptide integrity was confirmed. Bacteria colonies were grown in three steps, as described here. Small amounts of frozen bacteria from the glycerol stocks were grown on LB agar plates, which were pre-treated with ampicillin and chloramphenicol. Individual colonies

from the plate were selected and inoculated in 3 mL of Luria Broth (LB Broth, Appendix A) with both antibiotics (37°C, 225 RPM shaking). Finally, the bacteria colonies were added to 0.5 – 2.0 liters of LB broth with antibiotics. The variables tested for effect on bacteria growth were temperature, the presence of antibiotics, and the presence of the chemical isopropyl β -D-1-thiogalactopyranoside (IPTG).

2.4 Transcription Induction

Induction was performed using Isopropyl β -D-1-thiogalactopyranoside (IPTG, 1 mM), a molecular mimic of allolactose that triggers transcription of the lac operon. The pTAT vector contained the T7 lac promoter, allowing high levels of peptide expression upon induction. Bacterial expression levels were tested with variable bacteria growth temperatures and concentrations, the presence of chloramphenicol, the length of induction, and the type of broth, as will be discussed in the results.

2.5 Cell Lysis

Lysis was accomplished via sonication, which utilized ultrasonic waves to disrupt the cell membrane. Bacteria colonies were pelleted from their growth media (3000 RCF, 4°C), re-suspended in a lysis buffer and sonicated (Appendix B). The product solution was centrifuged (40,000 RCF), and the supernatant and precipitate were tested separately for peptide content. Highly soluble peptide expression was necessary before purification was possible; therefore, various conditions that effect cell lysis were tested. The parameters optimized to ensure maximum soluble recovery included utilization of a freeze-thaw protocol, and analyses of sonication levels, lysis buffers, and the bacteria growth temperature.

2.6 Peptide Purification

The first purification scheme utilized ammonium sulfate precipitation followed by ion-exchange chromatography. Urea was added to the lysate solution (6 M). The salt concentration was incrementally increased to 30%, 50%, and 70% of saturation (Appendix C). At low salt concentrations, hydrophobic peptides precipitated from solution. At each increment, the solution was centrifuged, the precipitate was separated and re-suspended into fresh buffer, the solution was dialyzed and a sample was analyzed for peptide content (Appendix C).

The fraction containing the decoy peptide was further purified using ion-exchange chromatography. The theoretical isoelectric point of the decoy was 11.5, resulting in a positively charged molecule at a pH of 8. The peptide solution (pH 8) was sent through anion exchange (positively charged resin), where negatively charged peptides were retained, but the peptide of interest flowed through. The flow through solution was then sent through cation exchange (negatively charged resin), where negative/ neutral peptides flowed through the column, but the peptide of interest was retained. The column was eluted by increasing the salt concentration along a gradient of a solution flowing through the column at a constant flow rate. Fractions were collected along the salt concentration gradient, and the effluent samples were tested for peptide content (Appendix D).

The second purification was accomplished by a nickel column. The histidine tag on the decoy peptide gave strong affinity for positively charged metal ions, such as nickel. The peptide solution was run through a column filled with nickel resin. The majority of cell contents passed through, but the peptide of interest was retained. The peptide was eluted by increasing the imidazole concentration of a flowing solution. The effluent samples were tested for peptide content (Appendix E).

2.7 Cellular Transduction

The peptide solutions were exposed at various concentrations to mouse tail VSMs and HEK-293 cells. Cell imaging was performed following transduction into VSMs with DAPI (blue) for nucleus visualization and AlexaFluor-488 (green) for the HA tag on the decoy peptide. Successful delivery was confirmed using HEK-293 cells by exposure of the peptide solution for 4 hours followed by washing of the cell membrane prior to Western blot analysis.

2.8 Western Blot and Coomassie Techniques

The presence of the decoy peptide in a given sample was detected using Western blot and Coomassie techniques (Appendix F). When performing a Western blot, the samples were run in an 18% poly-acrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The commercially available HA primary antibody was exposed to the membrane at a 1:1000 dilution, and the anti-mouse HRP secondary antibody was exposed at a 1:2000 dilution. To confirm the results, a custom synthesized α_{2C} -AR C-terminus primary antibody (Chotani, Mitra and Su) was used at a 1:1000 dilution with secondary anti-rabbit HRP at a 1:2000 dilution.

Coomassie staining was used directly on the poly-acrylamide gel, which gave the total peptide content. The results of Coomassie and Western blots were used in conjunction to determine the presence of peptide and a rough estimate for the quantity of expression.

Chapter 3: Results & Discussion

3.1 DNA Cloning

Successful DNA cloning is implied throughout the results section. Western blots giving signals at the expected size of the decoy peptide using both HA and α_{2C} -AR antibodies provided sufficient evidence to confirm that the decoy was present in the correct form.

3.2 Bacterial Transformation

The results from transformed bacteria samples can be seen in Figure 3. Glycerol stocks were made from Trials 1 and 3 because they showed the highest levels of expression. The glycerol stock from trial 3 was used to grow bacteria throughout the remainder of the report.

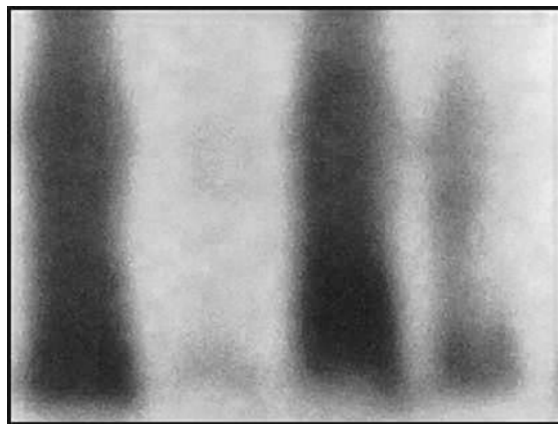


Figure 3: Transformed Bacteria Colonies

3.3 Bacteria Growth

Bacteria growth was studied at 37 °C, and the bacteria growth curves associated with various chemical additives can be seen in Figure 4. The logarithmic growth phase was recognized between a bacterial optical density of 0.2 angstroms (time = 0 hours) and 1.0 angstroms (time = 6 hours). Following the log phase, the bacteria growth rate was positive but to a much lesser extent. The legend to the right of the graph displays the chemical composition for four trials of bacteria growth. The letter “A” stands for the presence of ampicillin, “C” the presence of chloramphenicol, and “I” the presence of IPTG. The results indicated that chloramphenicol reduced the bacteria growth rate more than IPTG, and the bacteria grew fastest with only ampicillin.

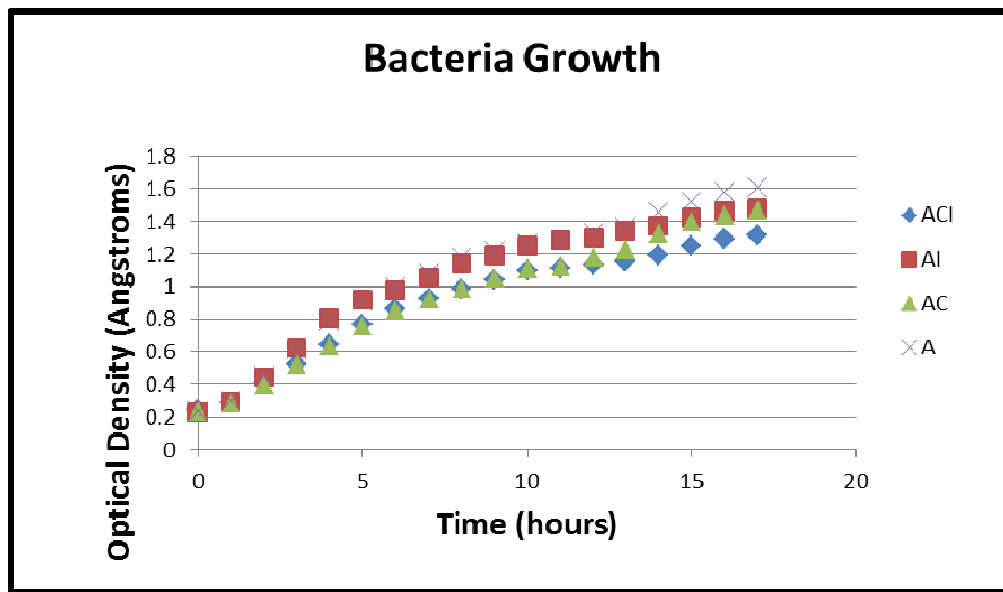


Figure 4: Bacteria Growth Curves

3.4 Transcription Induction

Transcription was induced using IPTG at bacteria concentrations of 0.230 angstroms and 1.20 angstroms in trials 1 and 2, respectively. Additionally, samples were induced with and without the presence of chloramphenicol. Figure 5 and Figure 6 show the results for the first and second trial, respectively.

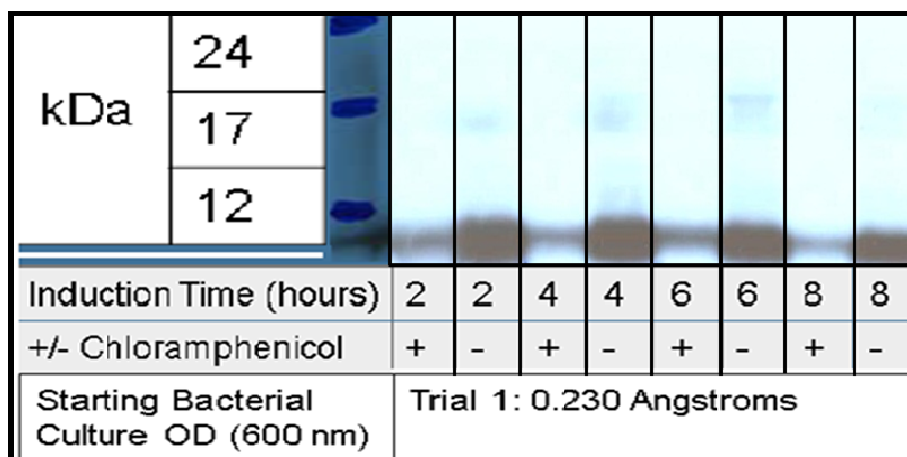


Figure 5: Induction at Low Bacterial Concentrations (+/-) Presence of Chloramphenicol

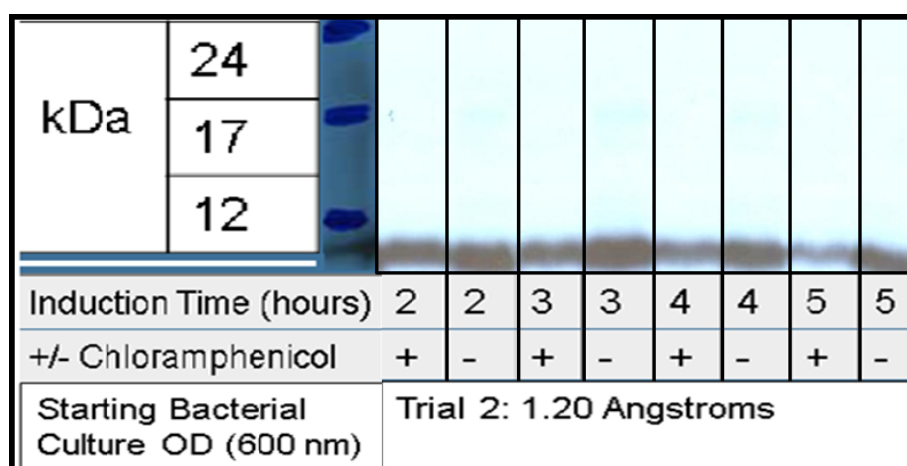


Figure 6: Induction at High Bacterial Concentrations (+/-) Presence of Chloramphenicol

Peptide expression increased when colonies were induced at low bacteria concentrations and when growth occurred without the presence of chloramphenicol. Maximum expression was shown between 4 and 6 hours following induction at 0.2 angstroms. The signal diminished between 6 and 8 hours after induction, indicating that the rate of peptide degradation was higher than the rate of formation after the log growth phase. In the second trial, the signal similarly diminished between 4 and 5 hours after induction, supporting the idea of a net decrease in peptide production beyond the log growth phase.

Levels of expression were also studied using various growth media. Initially, Luria Broth (LB) was used to grow bacteria (37°C); however, expression was not sufficient to produce high levels of soluble peptide. Therefore, Terrific Broth (TB) was substituted with otherwise identical

growth conditions. Levels of expression were examined after induction at 0.2 angstroms. Figure 7 displays the results using LB and TB growth media. The levels of expression were identical, and LB broth was used moving forward because of the higher cost associated with enriched TB growth media.

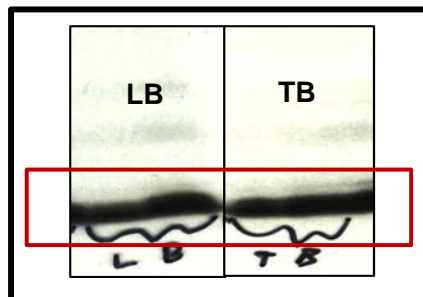


Figure 7: Induction in LB Broth and TB Broth

3.5 Cell Lysis

Heterologous proteins expressed in *E. Coli* may remain insoluble for two main reasons: insufficient cell lysis, or the formation of inclusion bodies around the peptide. As mentioned, soluble expression was critical for a successful purification; therefore, various lysis conditions were examined. To test for sufficient lysis, the utilization of a freeze thaw protocol prior to sonication was tested. Integration of a freeze/thaw technique would weaken the cell membrane, allowing for an effective lysis via sonication. The results for the freeze/thaw protocol can be seen in Figure 8. A “+” indicates a freeze/thaw protocol was utilized.

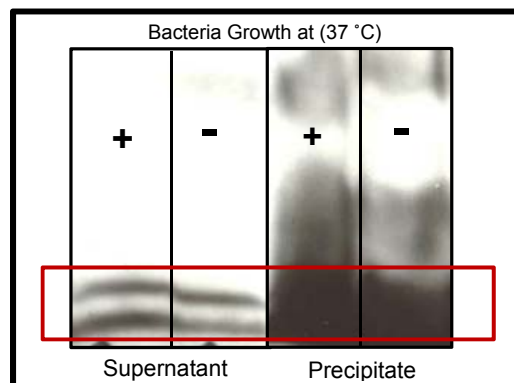


Figure 8: Soluble Expression (+/-) Utilization of Freeze Thaw

Comparing the levels of expression in the supernatant and precipitate during normal and freeze/thaw conditions indicated that an identical distribution of peptide resided in each sample whether or not the freeze/thaw procedure was included. Our conclusion was that the cells were sufficiently lysed without the freeze/thaw; therefore, the formation of inclusion bodies was further considered.

To test the formation of inclusion bodies, bacteria colonies were grown and induced at 37°, 30°, and 20°C. Soluble expression levels were qualitatively measured using Western blots. The results can be seen in Figure 9.

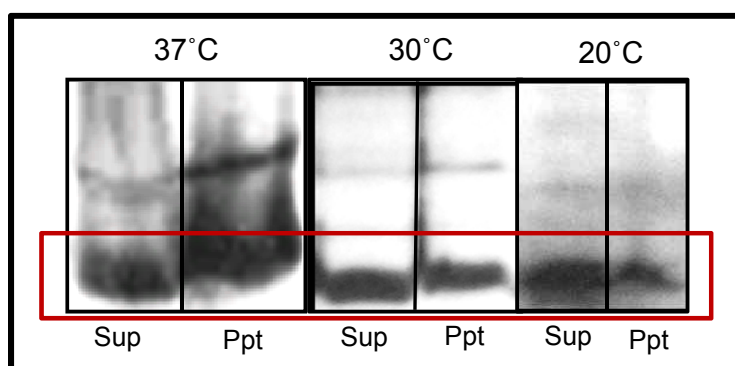


Figure 9: Soluble Expression at Various Temperatures

In general, high temperatures showed the highest level of overall expression but also the highest level of insoluble expression. Low temperatures showed an improved distribution of peptide in a soluble form; however, the total level of expression decreased. These results support the idea that inclusion bodies were the reason behind insoluble expression. Low temperatures decreased the thermodynamic stability of micelle-like formations; therefore increasing the effectiveness of sonication. However, decreased temperatures slowed bacteria growth; therefore decreasing total expression. A growth temperature of 30°C was utilized to balance between soluble and total expression.

3.6 Peptide Purification

3.6.1 Salt Precipitation

Upon achieving soluble peptide expression, ammonium sulfate precipitation was used to purify 50-70% of the undesired contents from solution. The results for Coomassie and Western blot techniques can be seen in Figure 10 below. The decoy peptide only showed a signal in the 50-70% fraction; therefore this fraction was further purified using ion exchange chromatography.

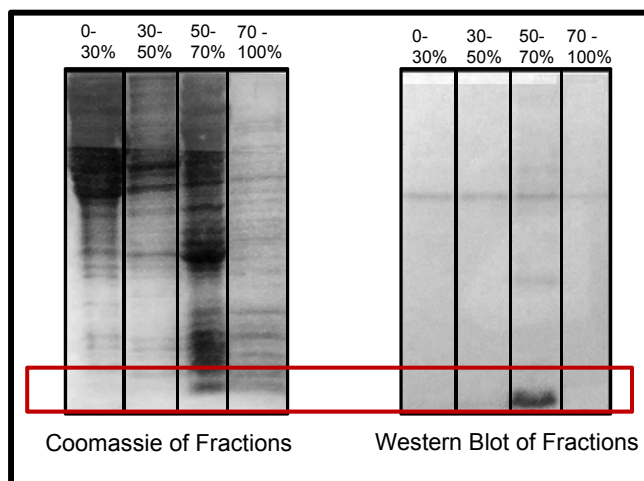


Figure 10: Ammonium Sulfate Precipitation Samples

3.6.2 Ion Exchange Chromatography

Ion exchange chromatography was utilized to yield a solution of 90-95% purity. The first column was filled with positively charged resin, and the results from the first column can be seen in Figure 11. As expected, the positively charged peptide was present in the flow through (FT).

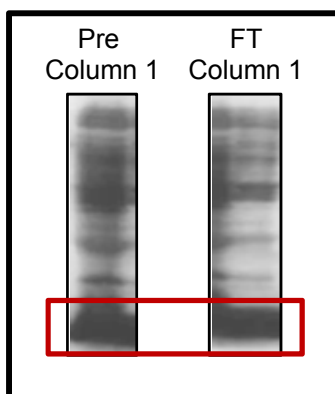


Figure 11: Ion Exchange Chromatography Column 1 Results

The flow through solution was sent through to a second, negatively charged column, and the peptide was retained in the resin. A solution with increasing salt concentration was sent through the second column to elute the peptide. Figure 12 shows the samples that were collected along the salt gradient.

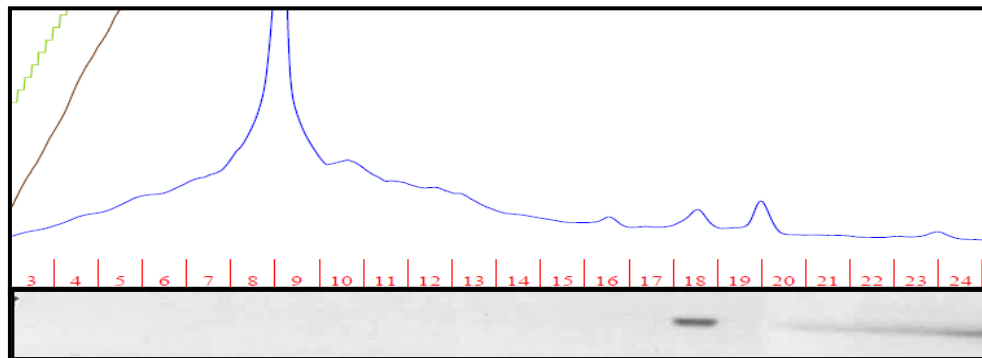


Figure 12: Ion Exchange Chromatography Column 2 Results

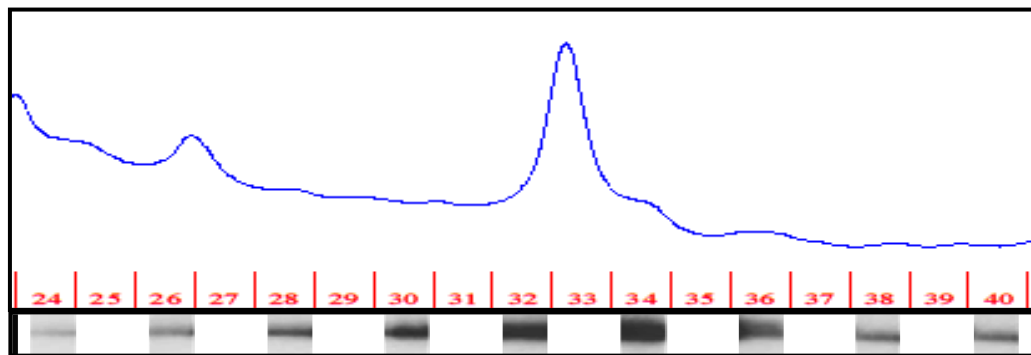


Figure 12: Ion Exchange Chromatography Column 2 Results

The blue line measured the detection of a tryptophan amino acid, indicating that a peptide had been eluted from the column. The collected fractions near an amino acid peak were tested for protein content using Western blots. The Western blot signal, which can also be seen in Figure 12, is aligned directly below the fraction being tested. The results indicated that several fractions surrounding the peak in sample “33” contained the peptide. These samples were dialyzed, lyophilized, and re-suspended in buffer so they could be tested for delivery to vascular smooth muscle cells.

3.6.3 Nickel Column

A nickel column was also used to purify the peptide. This technique directly purified the lysed bacteria solution, and the results can be seen in Figure 13.

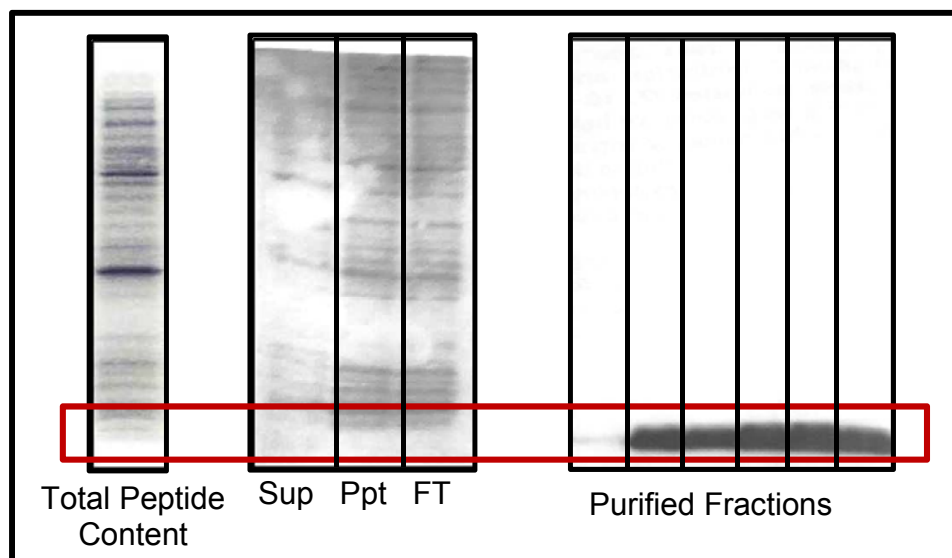


Figure 13: Nickel Column Purification Results

The total peptide content was equivalent to a combination of the supernatant and precipitate. Similarly, the total peptide content was equivalent to the flow through sample combined with the purified fractions. This procedure was much faster than the first purification method; however, the purity yield was not as high.

3.7 Cellular Transduction

The fractions purified by the nickel column were tested for delivery to mouse tail vascular smooth muscle cells (VSMs) and HEK-293 cells. The results can be seen in Figure 14 and Figure 15, respectively. The peptide was directly exposed to VSMs at a range of volumes, and these cells were tested for successful delivery using microscopic visualization. Figure 14 shows a strong positive signal in VSMs that were exposed to the decoy. Quantitatively, the signal was twice as intense for the cells containing the delivered peptide as the control.

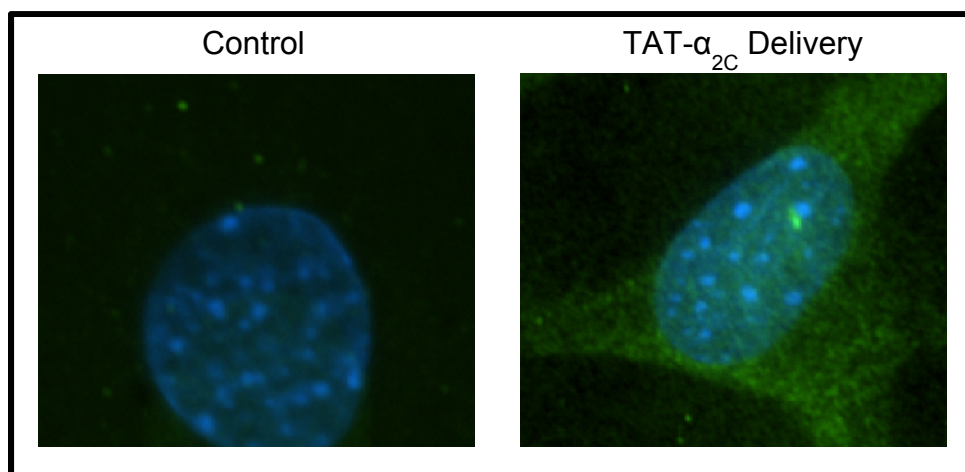


Figure 14: Microscopic Visualization of Peptide Delivery to Mouse Tail Artery VSMs

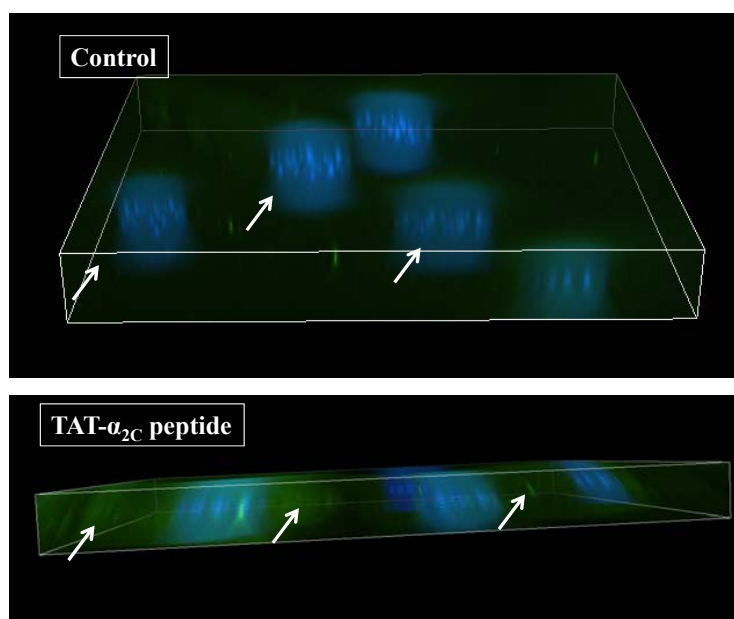


Figure 14 (continued): 3D-construction of images acquired in z-stacks, mouse tail artery VSMs. The arrows point to intracellular localization of signal in “TAT- α_{2C} peptide” cells compared to “Control” cells without peptide. Blue stain (DAPI) nucleus.

These results were confirmed by delivery to HEK-293 cells (Figure 15). The peptide was exposed to the cell membrane and allowed 4-6 hours for transduction. The membrane was washed; therefore any signal would indicate successful uptake by the cell. A strong signal was seen in a Western blot of the sample with decoy delivery; however, the control showed no signal.

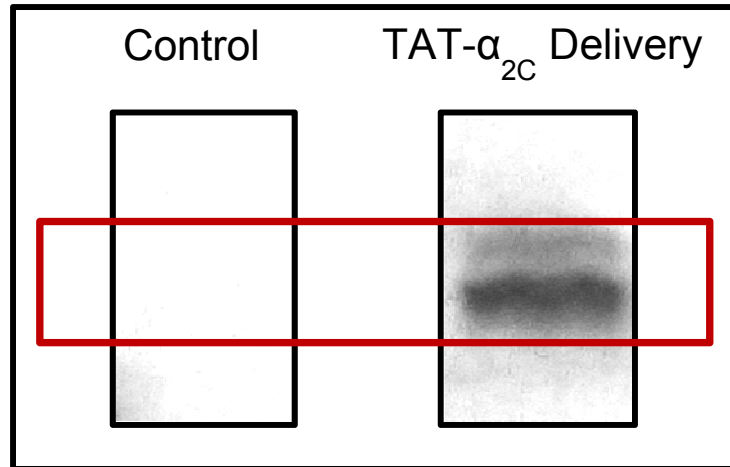


Figure 15: Western Blot of Peptide Delivery to HEK-293 Cells

Chapter 4: Conclusions and Recommendations

This thesis has shown the successful expression and purification of a peptide that is to be used for testing the mechanism of cold induced vascular constriction. Additionally, successful delivery of the peptide was demonstrated to mouse tail VSMs and HEK-293 cells.

Throughout the peptide expression process, several bacteria growth conditions were optimized. Growth at 30 °C limited the stability of inclusion bodies formed by bacteria defenses, therefore allowing soluble expression following sonication. Induction was performed strictly during the logarithmic bacteria growth phase, limiting the degradation of protein that occurred following non-ideal growth conditions. Utilization of freeze/thaw techniques and enriched growth media had negligible effects on peptide expression.

Peptide purification was performed using ammonium sulfate precipitation, ion exchange chromatography and nickel column techniques. During salt precipitation, the decoy peptide was present in a 50-70% ammonium sulfate saturation fraction. Ion exchange chromatography and nickel columns successfully purified the peptide, and the nickel column purified peptide tested for cell-delivery. Successful delivery to mouse tail VSMs and HEK-293 cells was confirmed using microscopic visualization, intensity comparisons, as well as Western blot techniques.

A cold exposure analysis using the decoy peptide is the next step to the process. As indicated, decoy will be used to tie up the binding sites to the actin binding filamin-2, effectively localizing the α_{2C} -AR in its sub-cellular location. Additionally, the effect of domain swapping the C-termini of the α_{2C} - and α_{2A} -ARs generating receptor chimera will provide additional insight on the role of the C-terminus in receptor trafficking.

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Appendices

Appendix A: Types of Broth

Table 1: Luria Broth Composition (Sigma-Aldrich)

Chemical	Concentration
Tryptone	10 g/L
Yeast Extract	5 g/L
NaCl	10 g/L

Table 2: Luria Broth Preparation Instructions

1. Suspend 25 g of Luria Broth base in 1 L distilled water
2. Autoclave for 15-20 minutes at 121 °C
3. Allow to cool to the touch, and add antibiotics

Table 3: Terrific Broth Composition (Sigma Aldrich)

Chemical	Concentration
Tryptone	12 g/L
Yeast Extract	24 g/L
K ₂ HPO ₄	9.4 g/L
KH ₂ PO ₄	2.2 g/L

Table 4: Terrific Broth Preparation Instructions

1. Suspend 47.6 g of Terrific Broth base, 8 mL glycerol in 1 L distilled water
2. Autoclave for 15-20 minutes at 121 °C
3. Allow to cool to the touch, and add antibiotics

Appendix B: Lysis Buffers and Conditions

Cells were pelleted from their growth media and re-suspended in a lysis buffer. Generally, in a solution of 1 L cells in growth media, the pelleted cells were re-suspended in 50 mL lysis buffer.

Table 5: Sucrose (STE) Lysis Buffer Composition

Chemical	Concentration
Sucrose	0.5 g/L
EDTA	1 mM
Tris-HCl pH 8.0	20 mM
Protease Inhibitors	1x

Table 6: Urea Lysis Buffer Composition

Chemical	Concentration
Urea	6 M
EDTA	1 mM
Tris-HCl pH 8	20 mM
NaCl	1 M
Protease Inhibitors	1x

Table 7: Sonication Procedure

1. Bacteria solution is pipetted into 50 mL conical vial
2. Optional addition of anti-foam suspension to top surface of bacteria solution
3. Solution is sonicated for 20 seconds on medium power
4. Solution is allowed to settle for 40 seconds
5. Steps 3-4 are repeated 5 additional times
6. Sonicate solution is ultracentrifuged at 40,000 RCF, 4 °C for 20 minutes

Appendix C: Ammonium Sulfate Precipitation Conditions

Table 8: Ammonium Sulfate Additions for Certain Saturation Levels (%) at 0 °C

Percent Saturation	Amount of Salt Addition
0-30%	16.6 g / 100 mL
30-50%	11.9 g / 100 mL
50-70%	12.7 g / 100 mL

Table 9: Dialysis Buffer Composition

Chemical	Concentration
Urea	6 M
EDTA	1 mM
Tris pH 8.0	20 mM

Table 10: Ammonium Sulfate Precipitation Procedure

1. Ammonium sulfate added to certain level of concentration (e.g. 30%)
2. Sample spun down at 40,000 RCF for 20 min
3. Precipitate separated from solution and re-suspended in Dialysis Buffer
4. Suspension homogenized if necessary
5. Steps 1-4 repeated to the supernatant solution at the next concentration (e.g. 50%)
6. All solutions dialyzed in a total of 1 L of fresh dialysis buffer for 12 hours
7. Step 6 repeated
8. All samples tested for peptide content

Appendix D: Ion Exchange Chromatography Purification Conditions

Table 11: Column Exchange Buffer

Chemical	Concentration
Urea	6 M
EDTA	1 mM
Tris pH 8.0	20 mM

Table 12: Column Elution Buffer

Chemical	Concentration
Urea	6 M
EDTA	1 mM
Tris pH 8.0	20 mM
NaCl	1 M

Table 13: Ion Exchange Chromatography Protocol

1. Buffers vacuum filtered and de-gassed
2. Peptide solution syringe filtered (44 um) and re-suspended in exchange buffer
3. Sample flow through column 1 (Q-seph)
4. Sample flow through column 2 (Source 15s)
5. Elution with buffer, fractions collected

Appendix E: Nickel Column Purification

Table 14: Sonication Buffer

Chemical	Concentration
NaCl	0.5 M
Tris HCl pH 7.4-8.5	20 mM
Imidazole	50 mM

Table 15: Elution Buffer 1

Chemical	Concentration
NaCl	0.5 M
Tris HCl pH 7.4	10 mM
Imidazole	0 mM

Table 16: Elution Buffer 2

Chemical	Concentration
NaCl	0.5 M
Tris HCl pH 7.4	20-50 mM
Imidazole	100 mM

Table 17: Elution Buffer 3

Chemical	Concentration
NaCl	0.5 M
Tris HCl pH 7.4	20-50 mM
Imidazole	500 mM

Table 18: Nickel Column Purification Procedure

1. Cells re-suspended in sonication buffer, sonicated, and spun down at 20,000 RCF.
2. 2.5 mL nickel resin added to plastic column with filter
3. Fresh sonication buffer flows through column at 5 times volume of resin (12 mL)
4. Column is capped, supernatant from sonication is poured into column, cap removed
5. After flow, column washed with 10 times column volume with elution buffer 1
6. Next, column is washed with 20 mL with fresh sonication buffer
7. Elution samples are checked for peptide content with color change in Bradford Reagent. To test with Bradford reagent, 30 uL reagent is added to 10 uL sample
8. Following elution with buffer 1, column is eluted with 2 times column volume with buffer 2 (5 mL). This is performed 1 mL at a time
9. Column is eluted with 1 mL buffer 3, repeated x6
10. Fractions are collected, tested with Bradford reagent, and utilized with Western Blot
11. Column is regenerated by washing with 10 times column volume with distilled water, 2 times the column volume with 0.5 M NaOH, and final wash with distilled water.
12. To increase peptide purity, NaCl concentration can increase to 1 M in all buffers

Appendix F: Peptide Visualization Using Western Blot and Coomassie

Table 19: SDS Lysis Buffer

Chemical	Concentration
Tris HCl pH 7.0	60 mM
SDS	2%

Table 20: 10 X Transfer Buffer (2L)

Chemical	Mass
Glycine	180 g
Tris Base	38.6 g
Water	2 L

Table 21: 1X Transfer Buffer (4L)

Chemical	Volume
10X Transfer Buffer	400 mL
Methanol	800 mL
Water	2800 mL

Table 22: TBS / Tween

Chemical	Concentration
Tris HCl pH 7.5	10 mM
NaCl	150 mM
Tween-20	0.1 %

Table 23: Blocking Solution

Chemical	Concentration
Non Fat Dry Milk	5% (5 g / 100 mL)
TBS / Tween	Solvent

Table 24: Western Blot and Coomassie General Protocol

1. Samples were mixed with 5X SDS loading dye in a 4:1 ratio
2. Samples were boiled at 75 °C for 10 minutes and spun down at 10,000 RCF for 2 minutes
3. Criterion, 18% tris-HCl gels were utilized with TGS Running Buffer
4. Current was set to 70 volts during stacking section, then 150 V until sufficient separation
5. Coomassie staining could be performed directly after running the gel. For a detailed protocol, see specific manufacturer instructions (give reference to manufacturer here)
6. For Western Blot visualization, gel peptides were transferred to the PVDF membrane using 1X transfer buffer. PVDF membrane was activated in methanol for 30 seconds prior to transfer.
7. Transfer current was set to 25 volts, which ran overnight.
8. Following Transfer, membrane was stained with Naphthol Blue Black to achieve preliminary peptide content visualization for successful transfer.
9. Following Naphthol staining, membrane was washed thoroughly with water, and then exposed to blocking solution for 1 hour
10. Membrane exposed to primary antibody solution for 1 hour with rocking

11. Membrane washed with TBS / Tween for 20 minutes, repeated twice (rocking)
12. Membrane exposed to secondary antibody solution for 1 hour (rocking)
13. Membrane washed with TBS / Tween for 20 minutes, repeated three times (rocking)
14. Chemiluminescent reaction performed on membrane using hydrogen peroxide, luminol, and peroxidase enzyme on secondary antibody
15. Membrane exposed to X-Ray film in dark room, and film processed.
16. Peptide content leads to signal on X-Ray film